ANTIFUNGAL EFFECT OF KAFFIR LIME LEAF EXTRACT ON SELECTED FUNGAL SPECIES OF PATHOGENIC OTOMYCOSIS IN IN-VITRO CULTURE MEDIUM

By

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ABSTRAK

Pendahuluan: Limau purut *(Citrus hystrix D.C)* telah digunakan sejak sekian lama dalam bidang pertanian dan perubatan alternatif. Daun limau purut telah terbukti berkesan terhadap kulat tertentu yang boleh menjangkiti manusia. Kajian ini bertujuan menilai kesan antikulat daun limau purut terhadap dua kulat patogenik otomikosis iaitu *Aspergillus niger* dan *Candida albicans*.

Metodologi: Kajian ini merupakan kajian makmal kawalan prospektif yang dijalankan di Universiti Sains Malaysia. Daun limau purut diproses menggunakan kaedah pengekstrakan Soxhlet dengan larutan 70% etanol dan air, kemudian dikeringkan untuk memperoleh serbuk yang pekat. Seterusnya, serbuk ekstrak tersebut telah dilarutkan dengan air steril untuk mendapatkan lima kepekatan berbeza iaitu 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml dan 3.125 g/ml. Kemudian ekstrak tersebut telah diuji berulang kali pada agar Sabouraud Dektrose yang telah disapukan kulat. Kaedah penyebaran agar telah digunakan dan zon perencatan telah diukur. Keputusan dicatatkan dan dianalisa menggunakan statistik.

Keputusan: Kedua-dua ekstrak larutan air dan alkohol daun limau purut menunjukkan perencatan pada pertumbuhan *Aspergillus niger* dan *Candida albicans*. *Candida albicans* menunjukkan kesan antikulat pada larutan 50 g/ml, 25 g/ml and 12.5 g/ml untuk kedua-dua ekstrak larutan air dan alcohol. Bagaimanapun, kadar perencatan pada pertumbuhan *Aspergillus niger* cuma pada larutan 50 g/ml untuk kedua-dua ekstrak larutan air dan alkohol. Kesan antikulat yang ditunjukkan ke atas *Aspergillus niger* adalah lebih baik dalam larutan air jika dibandingkan dengan larutan alkohol dengan signifikasi $p<0.001$. Sebaliknya, kesan antikulat yang ditunjukkan ke atas *Candida albicans* adalah lebih baik dalam ekstrak larutan alkohol jika dibandingkan dengan ekstrak larutan air dengan signifikasi $p<0.001$. Nilai 'Minimum inhibitory concentration(MIC)' untuk ekstrak larutan air terhadap *Candida*

albicans ialah 12.0.2 g/ml berbanding terhadap *Aspergillus niger* 47.86 g/ml, manakala ekstrak larutan alkohol terhadap *Candida albicans* ialah 10.23 g/ml berbanding terhadap *Aspergillus niger* 48.97 g/ml.

Kesimpulan: Daun limau purut terbukti mempunyai kesan antikulat terhadap *Aspergillus niger* dan *Candida albicans.* Perbandingan menunjukkan bahawa kesan antikulat terhadap *Candida albicans* adalah lebih baik berbanding *Aspergillus niger* dalam kedua-dua ekstrak larutan air dan alkohol daun limau purut.

ABSTRACT

Introduction: Kaffir lime *(Citrus hystrix D.C)* has been used for a long time in agricultural and alternative medicine. Kaffir lime leaf (KLL) had been proved effective against certain fungi that could infect human body. This study aims to demonstrate the antifungal effect of KLL extract on pathogenic otomycosis species, particularly *Aspergillus niger* and *Candida albicans.*

Methods: This is a laboratory-controlled prospective study conducted in Universiti Sains Malaysia. KLL was extracted via Soxhlet extraction method by using 70% ethanol and aqueous, then evaporated using rotary evaporator to a thicker compound. The concentrated extract then freeze dried to obtain powdered form which was diluted to establish five different concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Sabouraud Dextrose agar (SDA) lawned with tested fungal isolates were inoculated with the extracts using well-diffusion method. Zone of inhibition was measured followed by minimum inhibitory concentration (MIC).

Results: There were zone of inhibition for both aqueous and alcohol KLL extracts on *Aspergillus niger* and *Candida albicans* growth. *Candida albicans* has antifungal activities in concentrations of 50 g/ml, 25 g/ml and 12.5 g/ml for both aqueous and alcohol KLL extracts. However, zone of inhibition for *Aspergillus niger* was obtained only in 50 g/ml concentration for both aqueous and alcohol KLL extracts. Statistically antifungal activity of *Aspergillus niger* is better by aqueous KLL extracts as compared to alcohol KLL extract with significance difference $p<0.001$. In contrast, antifungal activity of *Candida albicans* is better in alcohol extracts as compared to aqueous extract with significance $p<0.001$. The MIC of KLL aqueous extract against *Candida albicans* was 12.02 g/ml, KLL alcohol extract against

Candida albicans was 10.23 g/ml, KLL aqueous extract against *Aspergillus niger* was 47.86 g/ml and KLL alcohol extract against *Aspergillus niger* was 48.97 g/ml.

Conclusion: KLL has significant antifungal effect towards pathogenic fungi species causing otomycosis, particularly *Aspergillus niger* and *Candida albicans*. The antifungal effect of both aqueous and alcohol KLL are seen more on *Candida albicans* as compared to *Aspergillus niger.*

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Kaffir lime (*Citrus hystrix D.C*) has been used for a long time in Asian cuisine, agriculture and folk medicine. This tropical fruit belongs to the family *Rutaceae* and also known as kieffer lime, leech-lime, Thai bergamot, limau purut or magrood.[1] Preclinical studies have shown that the phytochemicals compound of Kaffir lime leaves and fruit have antioxidant activity,[2] free radical scavenging ability,[2] anti inflammatory activity,[3] antibacterial activity,[4] antifungal activity,[5] and anticarcinogenic activity.[6]

Phytochemical studies has shown that citronellal is the main compound found in kaffir lime leaf.[7] The other chemical compounds found in kaffir lime leaf are a-pinene, camphene, b-pinene, limonene, copaene, linalool, b-cubebene, isopulegol, caryophyllene, citronellyl acetate and citronellol.[7] The (–)-(S)-enantiomer of citronellal makes up to 80% of the leaf oil from kaffir lime leaf and is the compound responsible for its characteristic aroma. Research shows that citronellal has strong antifungal qualities.[8]

Increase of otomycosis incidence has been linked to the extensive use of antibiotic eardrops, widespread use of steroids, broad spectrum antibiotics, and chemotherapeutic agents.[13] The fungal isolates in otomycosis are *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Candida albicans*, *Mucor* spp. and *Pencillium* spp.[13] Recent clinical mycological study mentioned that the most common fungal isolates in otomycosis belonged to the species of *Aspergillus* (82%) with *Aspergillus niger* (37%) was the most common isolate followed by *Aspergillus flavus* (12%).[14] Next common group was *Candida* species, accounting for 13% of total isolates.[14] Similarly, Malaysian data on fungal isolates in otomycosis revealed that *Aspergillus niger* was the commonest isolated fungi (71%) and *Candida albicans* was the second most common (23.4%).[15]

Aspergillus niger colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. This species of filamentous fungi produces several secondary toxic metabolites such as 3-nitropropionic acid and ochratoxin A.[16] In contrast, *Candida albicans* has the capability to form biofilm.[17] The importance of this character is a reduced susceptibility to the host immune system and to conventional antifungal drug therapy.[17] The diagnosis of otomycosis is clinical and accompanied by microbiological confirmation. Otoscopic examination of ear canal findings differs according to type of fungus. In *Aspergillus niger*, blackish fungal hypae, mycelia and wet paper appearance. In early stage it may resemble *Candida albicans* with cotton-wool like appearance or debris assembling furry white blanket.

Eradication of this entity remains a challenge to medical practitioners especially the otorhinolaryngologist. Until today, there has been no standardised treatment for otomycosis.[18] Articles reviewed on ototopical antifungal and otomycosis noted that not many data are available regarding safety of use of ototopical medication especially in presence of tympanic perforation.[19] In cases of perforated tympanic membrane, the ototopical antifungal should not be used because it can cause inflammation and granulation tissue in the middle ear. Besides that, the topical antifungal can readily reach the cochlea by diffusion through the round window. If the agent has ototoxic property, temporary and permanent electrophysiology changes within inner ear or morphological injury to stria vascularis, hair cells and supporting cells of organ of corti may occur. This situation can leads to ototoxicity and sensory neural hearing loss.[18, 19]

When choosing the correct topical antifungal drugs, certain factors should be considered such as water soluble, low risk of ototoxicity, low allergic effect, a broad spectrum antimycotic drug, suitable for application on paediatric patients, and commercially available.[19] Therefore, until today there is no topical antifungal that can provide all the mentioned factors. These

opens door for the option of safer alternative medication such as herbal or rather less toxic topical agents probably derived from kaffir lime leaf. There were very limited publications on antifungal effect of kaffir lime leaf especially to *Candida albicans* and *Aspergillus niger*. Hence, in this study, we are analysing the antifungal properties of kaffir lime leaf extracts towards pathogenic otomycosis, particularly *Aspergillus niger* and *Candida albicans.*

CHAPTER 2

OBJECTIVES OF THE STUDY

2.1 General Objective

To study the antifungal effect of kaffir lime (*Citrus Hystrix*) leaf extracts on *Aspergillus niger* and *Candida albicans*.

2.2 Specific Objectives

1. To determine inhibitory efficacy of different concentration of kaffir lime leaf water and alcohol extracts on *Aspergillus niger*
2. To determine inhibitory efficacy of different concentration of kaffir lime leaf water and alcohol extracts on *Candida albicans*
3. To compare antifungal activity between water and alcohol kaffir lime leaf extract against *Aspergillus niger*
4. To compare antifungal activity between water and alcohol kaffir lime leaf extract against *Candida albicans*

2.3 Research Hypothesis

Kaffir lime leaf extracts has antifungal effect against *Aspergillus niger* and *Candida albicans* at different concentrations.

CHAPTER 3

MANUSCRIPT

3.1 TITLE: ANTIFUNGAL EFFECT OF KAFFIR LIME LEAF EXTRACT ON SELECTED FUNGAL SPECIES OF PATHOGENIC OTOMYCOSIS IN IN-VITRO CULTURE MEDIUM

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3.2 ABSTRACT

Background: Kaffir lime *(Citrus hystrix D.C)* has been used for a long time in agricultural and alternative medicine. Kaffir lime leaf (KLL) had been proved effective against certain fungi that could infect human body.

Objective: To study the antifungal effect of kaffir lime (*Citrus Hystrix*) leaf extracts on *Aspergillus niger* and *Candida albicans*.

Methods: This is a laboratory-controlled prospective study conducted in Universiti Sains Malaysia. KLL was extracted with 70% ethanol and aqueous via Soxhlet extraction method. The concentrated extract then freeze dried to obtain powdered form which was diluted to establish five different concentrations of 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Sabouraud Dextrose Agar (SDA) lawned with tested fungal isolates were inoculated with the extracts using well-diffusion method. Zone of inhibition was measured followed by minimum inhibitory concentration (MIC).

Results: There were presences of zone of inhibition for both aqueous and alcohol KLL extracts on *Aspergillus niger* and *Candida albicans* growth. KLL aqueous extract have bigger mean of inhibition as compared to alcohol extract with significance $p<0.001$ against *Aspergillus niger*. In contrast, KLL alcohol extract have bigger mean of inhibition as compared to aqueous extract with significance $p<0.001$ against *Candida albicans*. The MIC of KLL aqueous extract against *Candida albicans* was 12.02 g/ml, alcohol extract against *Candida albicans* was 10.23 g/ml, KLL aqueous extract against *Aspergillus niger* was 47.86 g/ml and alcohol extract against *Aspergillus niger* was 48.97 g/ml.

Conclusion: KLL has significant antifungal effect towards pathogenic fungi causing otomycosis, particularly *Aspergillus niger* and *Candida albicans*. Statistically antifungal activity of *Aspergillus niger* is better in aqueous extracts as compared to alcohol extract with significance $p<0.001$. In contrast, antifungal activity of *Candida albicans* is better in alcohol extracts as compared to aqueous extract with significance $p<0.001$. *Candida albicans* was inhibited better as compared to *Aspergillus niger* in both aqueous and alcohol KLL extract.

KEYWORDS

Otomycosis, Kaffir lime, *Citrus hystrix*, *Aspergillus niger*, *Candida albicans*

3.3 INTRODUCTION

Kaffir lime (*Citrus hystrix D.C*) has been used for a long time in folk medicine.[1] Preclinical studies have shown that the phytochemicals compound of kaffir lime leaf and fruit have antioxidant activity,[2] free radical scavenging ability,[2] antiinflammatory activity,[3] antibacterial activity,[4] antifungal activity,[1,5] and anticarcinogenic activity.[6]

Phytochemical studies has shown that citronellal is the main compound found in kaffir lime leaf.[7] The other chemical compounds found in kaffir lime leaf are a-pinene, camphene, b-pinene, limonene, copaene, linalool, b-cubebene, isopulegol, caryophyllene, citronellyl acetate and citronellol.[8] Research shows that citronellal has strong antifungal qualities.[9]

Otomycosis or fungal infection of the external auditory canal can be caused by *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Candida albicans*, *Mucor* spp. and *Pencillium* spp.[10] Malaysian data on fungal isolates in otomycosis revealed that *Aspergillus niger* was the commonest isolated fungi (71%) and *Candida albicans* was the second most common (23.4%).[11]

Aspergillus niger able to produce several secondary toxic metabolites such as 3-nitropropionic acid and ochratoxin A.[12] In contrast, cell surface hydrophobicity, biofilm formation and adhesion composite resin process are the important factors in defence mechanism of *Candida albicans.*[13] The importance of this character is a reduced susceptibility to the host immune system and to conventional antifungal drug therapy.[13]

Eradication of this entity remains a challenge to medical practitioners especially the otorhinolaryngologist. Until today, there has been no standardised treatment for otomycosis.[14] Articles reviewed on ototopical antifungal and otomycosis noted that less data available regarding safety of use of ototopical medication especially in presence of tympanic perforation.[15] In a case of perforated tympanic membrane, the topical antifungal can readily

reach cochlea by diffusion through the round window. If the agent has ototoxic property, temporary and permanent electrophysiology changes within inner ear or morphological injury to stria vascularis, hair cells and supporting cells of organ of corti may occur. This situation can leads to ototoxicity and sensory neural hearing loss.[14, 15]

When choosing the correct topical antifungal drugs, certain factors should be considered such as water soluble, low risk of ototoxicity, low allergic effect, a broad spectrum antimycotic drug, suitable for application on pediatric patients, and commercially available.[15] There were very limited publications on antifungal effect of kaffir lime leaf especially to *Candida albicans* and *Aspergillus niger*. Hence, in this study, we are analysing the antifungal properties of KLL extracts towards pathogenic otomycosis, particularly *Aspergillus niger* and *Candida albicans.*

3.4 METHODOLOGY

Study design

This is a laboratory-controlled prospective study. It was fully conducted under a well-controlled environment in the Medical Microbiology and Pharmacology laboratories in the School of Medical Sciences, Universiti Sains Malaysia. This study has been approved by the Human Ethics Committee of Universiti Sains Malaysia.

Extracts from KLL will be isolated using two different solvents which is aqueous and alcohol. Each extracts are diluted into five different concentrations and test to five replicates of fungal cultures. Thus, one type of fungal culture is used for five separate concentrations and one fungal culture test with two types of solvents which ended up to ten samples. Therefore, 50 samples are tested for five replicates in standard laboratory settings. Total sample for two types of fungal culture was 100 samples.[16]

Sample preparation method

KLL were collected from a single area, prepared for USM herbarium identification (voucher reference number 11538). The remaining KLL were washed with distilled water and dried in oven at 45^{o}C for two days. Dried KLL leaves should maintain the original green colour. Then, the dried leaves were grinded using leaf grinder machine to smaller course powder form and stored in tightly sealed glass container.

Sample extraction method

The method of choice for extraction was the Soxhlet extraction by using Soxhlet apparatus. This uses a solvent for extraction and at completion the solvent will be fully removed. We used two types of solvent which was aqueous and ethanol 70%. The dried powder form of the tested leaves was inserted into the Soxhlet thimble and closed with white thin gauze. The thimble was inserted into the Soxhlet main chamber and closed. The solvent chamber was filled with ethanol 70% 1 litre, and attached to Soxhlet apparatus. Solvent chamber should not be overfilled and the volume of solvent in the vessel should be 3 to 4 times the volume of the Soxhlet chamber. The solvent chamber was heated and solvent vapour travels up a distillation arm, and fills into the main Soxhlet chamber. The condenser ensures that any solvent vapour cools, and drips back down into the chamber housing the powder. Some of the desired compound was then dissolved in the warm solvent. When the Soxhlet chamber was almost full, the chamber was automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This extraction allowed for 4-6 cycles. By 6th cycle, the solvent chamber was dark in colour and Soxhlet chamber was clearer. The solvent with desired compound was extracted and in solvent chamber. The extracted leaves in alcohol solvent then evaporated with rotary evaporator. The volume of extract was concentrated till 50 ml and inserted into multiple sterile containers. It was freeze dried to avoid further heat

damage on a freeze dryer machine. For water solvent, same process was done whereby the ethanol was replaced with distilled water in solvent chamber.

This powder form extracts were used to establish different concentrations as required in this study. The concentrations required were 50 g/ml, 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. Initial 100 g mixed with two ml of distilled water to produce 50 g/ml concentration. Then one ml from the 50 g/ml concentration taken and distilled water added till two ml level to make 25 g/ml concentration. Similarly, one ml was taken from 25 g/ml concentration to make up 12.5 g/ml. The dilution continued till 3.125 g/ml concentration. The left over extracts was discarded and a new preparation done for new replicates.

Preparation of *in vitro* culture medium

The fungal isolates were taken from archives of microbiology laboratory in School of Medical Sciences, Universiti Sains Malaysia, which already been identified earlier from patients of otorhinolaryngology clinic, Hospital USM. Both *Candida albicans* and *Aspergillus niger* was used as tested fungi. *Candida albicans* from SDA plates were suspended in sterile distilled water and adjust to 10^6 cells with colony forming units (CFU)/ml^9 (0.5 McFarland standard). Nephelometer was used to adjust the turbidity of fungal suspensions so that the number of fungal was within a given standard McFarland range. Similar process done for *Aspergillus niger*.

The suspended sterile fungal organism labelled and used for the next step to lawn and prepare for testing in SDA plates. Within 15 minutes of diluting the organism, sterile swab was dipped into the properly adjusted inoculums of tested fungal organism. The sterile swab was slightly lifted up, and then the swab was firmly rotated several times against the upper inside wall of the tube to express excess fluid. The plate was open slightly to lawn the fungal organism. Later, by using glass pipette, four wells were created in four quadrants.

Initiation of *in vitro* test and data collection

The SDA plate was kept lid side up in a 30°C incubator. Before inserting in an incubator, micropipette was used to drop 100 microliter of extracts into wells. The upper quadrant well, the aqueous KLL extract was inserted with its aqueous control at opposite site. The lower quadrant well, the alcohol extracts was inserted with its alcohol control at opposite site. This was done using different concentration which was diluted prior to this. Five different replicates were done. The plates were examined every day to make sure no spillage or growth of other organism. The measurement was done on third day whereby this was the perfect time to visualize the margin of inhibition. After the third day, there was overgrowth especially the *Aspergillus niger* which may jeopardize the safety of the staff. Measurement was done of zones showing complete inhibition by gross visual inspection.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration measured by agar diffusion method to determine MIC of each tested KLL extracts. After measurement of all the result, a scattered plot graph X^2 Versus Log Concentration was plotted. A linear line represent the mean value was drawn and the area where X^2 equal to zero was taken for the MIC level of the extract. Antilog of the selected value was mathematically identified as MIC. The X value represent the zone of inhibition diameter subtract the well diameter and divided by two.

3.5 RESULTS

Zone of inhibition which is the clear zone where growth of fungal does not occurred was measured for each tested KLL extracts. There were zone of inhibition for both aqueous and alcohol KLL extracts after three days of incubation on *Candida albicans*. Similarly, *Aspergillus niger* growth was inhibited in aqueous and alcohol KLL extracts. However, not all concentration of extracts effectively inhibited the growth in both pathogens. *Aspergillus niger* was inhibited only in the highest concentration at 50 g/ml in both aqueous and alcohol KLL extracts. All samples revealed negative control (wells contain either only alcohol or only aqueous) showed no zone of inhibition against *Aspergillus niger* and *Candida albicans*.

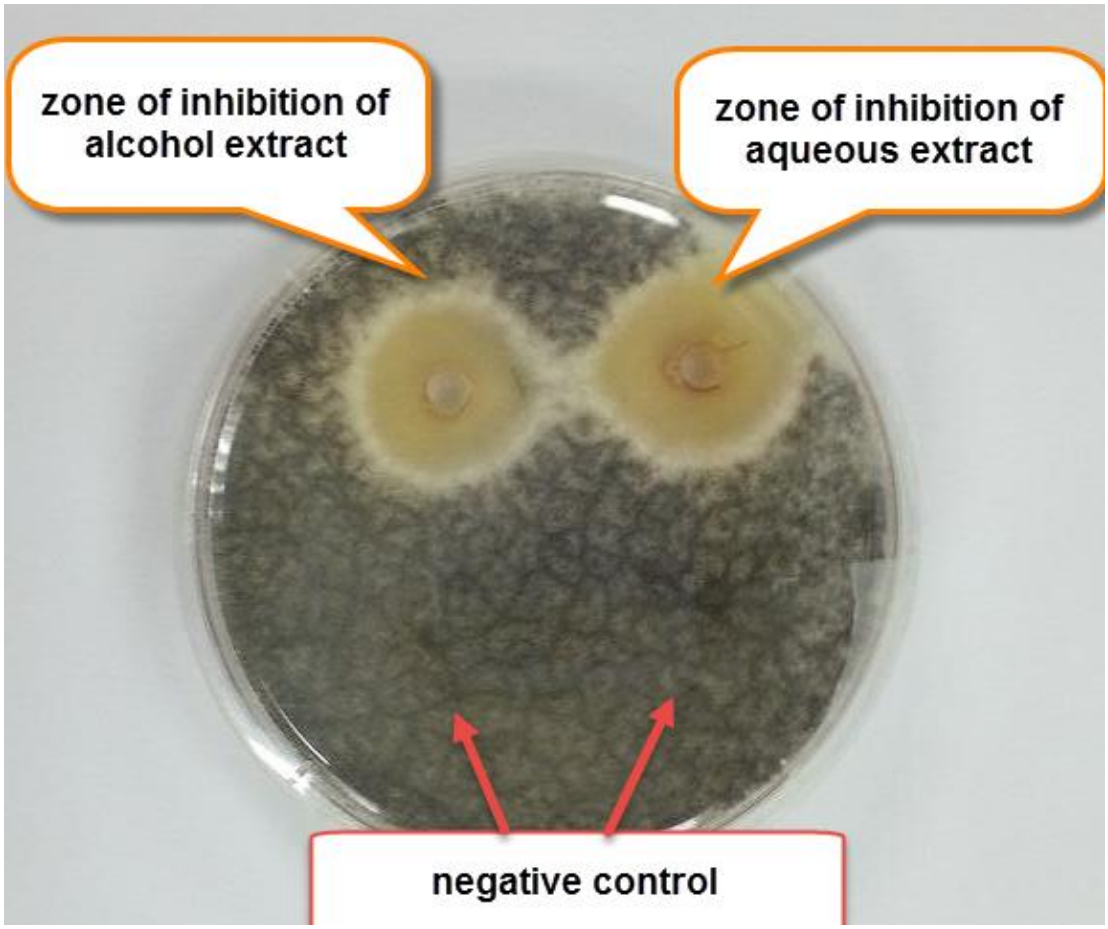

Figure 1: Positive zone of inhibition against *Aspergillus niger* with negative control in kaffir lime leaf aqueous and alcohol extracts at 50 g/ml concentration.

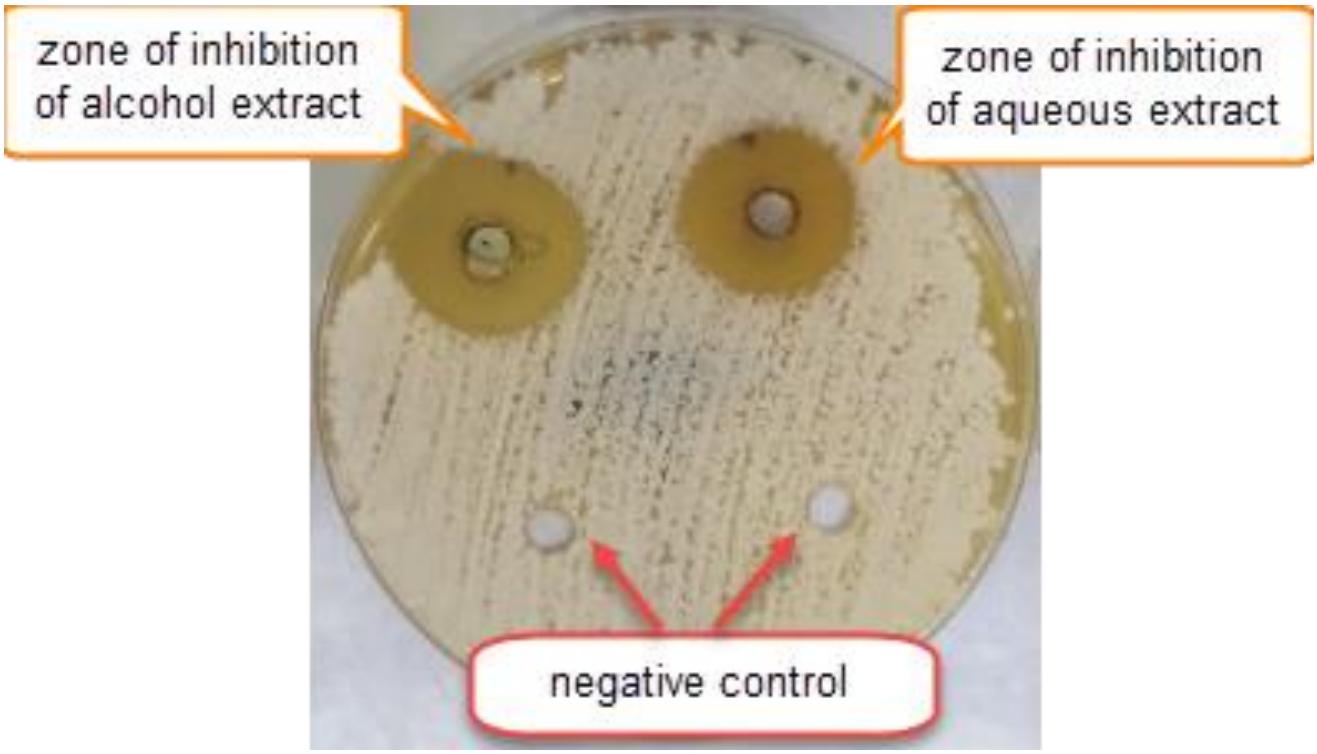

Figure 2: Positive zone of inhibition against *Candida albicans* with negative control in aqueous and alcohol extract at 12.5 g/ml concentration.

Inhibitory efficacy of kaffir lime leaf on *Aspergillus niger*

Aspergillus niger showed sensitivity to both aqueous and alcohol KLL extracts (Table 1) Growth of *Aspergillus niger* inhibited in KLL alcohol and aqueous extract in concentration of 50 g/ml. However the growth was resistance to concentration from 25 g/ml, 12.5 g/ml, 6.25 g/ml and 3.125 g/ml. One replicate of samples shown in Figure 3 at different concentrations whereby aqueous KLL extract tested at right upper quadrant of petri dish and alcohol KLL extract tested at left upper quadrant of each petri dish. Mean zone of inhibition is higher in extract concentration of aqueous compared to alcohol.

Table 1: Measurement of zone of inhibition of alcohol and aqueous kaffir lime leaf extracts on *Aspergillus niger* growth at five different concentrations.

Extracts	**Concentration (g/ml)**	**Zone of Inhibition (mm)**					**Mean (mm)**
		R1	**R2**	**R3**	**R4**	**R5**	
Aqueous	50.000	14	13	14	14	15	14.00
	25.000	0	0	0	0	0	.00
	12.500	0	0	0	0	0	.00
	6.250	0	0	0	0	0	.00
	3.125	0	0	0	0	0	.00
Alcohol	50.000	10	12	12	11	12	11.40
	25.000	0	0	0	0	0	.00
	12.500	0	0	0	0	0	.00
	6.250	0	0	0	0	0	.00
	3.125	0	0	0	0	0	.00

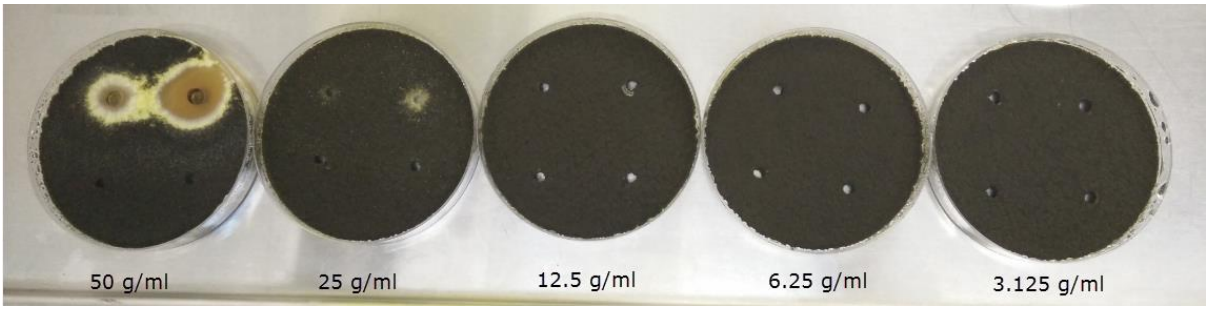

Figure 3: One replicate of samples showing zone of inhibition of kaffir lime leaf extracts on *Aspergillus niger* growth at 5 different concentrations.

Inhibitory efficacy of kaffir lime leaf on *Candida albicans*

Candida albicans showed sensitivity to both aqueous and alcohol KLL extracts (Table 2). Growth of *Candida albicans* were inhibited in KLL alcohol and aqueous extract concentrations of 50 g/ml, 25 g/ml and 12.5 g/ml. One replicate of samples at different concentrations whereby aqueous KLL extract tested at left upper quadrant of petri dish and alcohol KLL extract tested at right upper quadrant of each petri dish (Figure 4). Mean zone of inhibition increase in proportion to extract concentration.

Table 2: Measurement of zone of inhibition of alcohol and aqueous kaffir lime leaf extracts on *Candida albicans* growth at five different concentrations.

Extracts	**Concentration (g/ml)**	**Zone of Inhibition (mm)**					**Mean (mm)**
		R1	**R2**	**R3**	**R4**	**R5**	
Aqueous	50.000	26	27	28	27	28	27.20
	25.000	16	17	18	17	18	17.20
	12.500	10	11	12	10	12	11.00
	6.250	0	0	0	0	0	.00
	3.125	0	0	0	0	0	.00
Alcohol	50.000	29	30	31	29	31	30.00
	25.000	23	23	24	23	24	23.40
	12.500	13	14	15	13	14	13.80
	6.250	0	0	0	0	0	.00
	3.125	0	0	0	0	0	.00

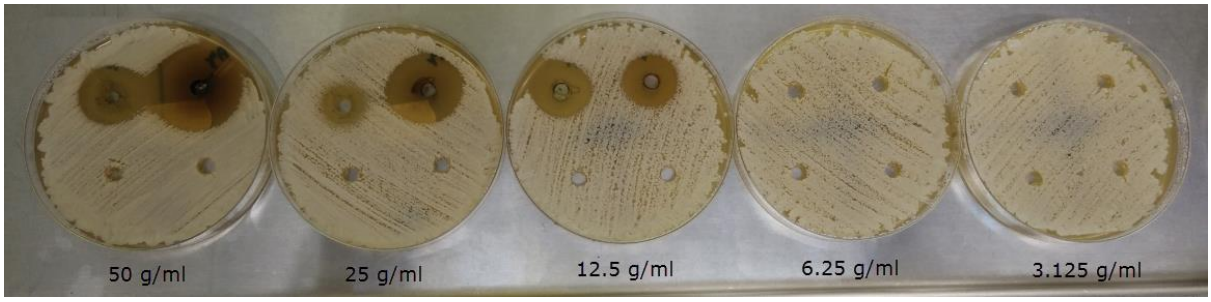

Figure 4: One replicate of samples showing zone of inhibition of kaffir lime leaf extracts on *Candida albicans* growth at five different concentrations.

One-way ANOVA showed significant mean differences between groups of concentration for aqueous KLL extracts (Table 3). Mean zone of inhibition of concentration 50 g/ml was 27.20 mm, 25 g/ml was 17.20 mm and 12.5 g/ml was 11.00 mm with significant difference between the three groups $p<0.001$. Post-hoc comparison showed significant difference 50 g/ml & 25 g/ml ($p< 0.001$), 50 g/ml & 12.5 g/ml ($p<0.001$) and 25 g/ml & 12.5 g/ml ($p<0.001$) (Table 4). The higher KLL aqueous concentrations gave high effect to mean of inhibition of growth of *Candida albicans*.

Table 3: Comparison of mean zone of inhibition against *Candida albicans* among three concentration groups in aqueous kaffir lime leaf extracts.

Concentration of extracts (g/ml)	**Mean (SD)**	**F-statistic (df)**	**p-value**
50.0	27.20(0.84)	417.58(2)	<0.001
25.0	17.20(0.84)		
12.5	11.00(1.00)		

One-Way ANOVA test was applied

Table 4: Post-hoc comparison of mean difference of zone of inhibition against *Candida albicans* among three concentration groups in aqueous kaffir lime leaf extracts.

Concentration of extracts (g/ml)	**Mean difference (95% Confidence Interval)**	**p-value**
50.0 vs 25.0	10.00 (8.42, 11.58)	<0.001
50.0 vs 12.5	16.20 (14.62, 17.78)	<0.001
25.0 vs 12.5	6.20 (4.62, 7.78)	<0.001

aScheffe test was applied

One-way ANOVA also showed significant mean differences between groups of concentration for alcohol KLL extracts against *Candida albicans* (Table 5). Mean zone of inhibition of concentration 50 g/ml was 30.00 mm, 25 g/ml was 23.40 mm and 12.5 g/ml was 13.80 mm with significant difference between the three groups $p< 0.001$. Post- hoc comparison showed significant difference 50 g/ml & 25 g/ml ($p< 0.001$), 50 g/ml & 12.5 g/ml ($p<0.001$) and 25 g/ml & 12.5 g/ml ($p<0.001$) (Table 6). Therefore, statistically one way ANOVA showed significant mean differences between group of concentration for both alcohol and aqueous KLL extracts against *Candida albicans* with p value < 0.001.

Table 5: Comparison of mean zone of inhibition against *Candida albicans* among three concentration groups in alcohol kaffir lime leaf extracts.

Concentration of extracts(g/ml)	**Mean (SD)**	**F-statistic (df)**	**p-value**
50.0	30.00(1.00)	497.70(2)	<0.001
25.0	23.40(0.55)		
12.5	13.80(0.84)		

One-Way ANOVA test was applied

Table 6: Post-hoc comparison of mean difference of zone of inhibition against *Candida albicans* among three concentration groups in alcohol kaffir lime leaf extracts.

Concentration of extracts (g/ml)	**Mean difference (95% Confidence Interval)**	**p-value**
50.0 vs 25.0	6.60 (5.16, 8.04)	<0.001
50.0 vs 12.5	16.20 (14.76, 17.64)	<0.001
25.0 vs 12.5	9.60 (8.16, 11.04)	<0.001

aScheffe test was applied

Comparison on antifungal activity of kaffir lime leaf aqueous and alcohol extract against *Aspergillus niger*

Comparison between antifungal activity of KLL aqueous and alcohol extract against *Aspergillus niger* demonstrated that antifungal activity in aqueous KLL extract is better than alcohol KLL extract. In concentration 50 g/ml, zone of inhibition of aqueous KLL extract was bigger as compared to alcohol KLL extract (Table 7). Independent t-test showed aqueous extracts has bigger mean of inhibition as compared to alcohol extract with significant difference of $p<0.001$ against *Aspergillus niger* (Table 7). Ninety five percent confidence interval of mean difference of inhibition does not include zero. Therefore statistically antifungal activity of *Aspergillus niger* is better in aqueous KLL extract than alcohol extract.

Table 7: Comparison of mean of zone inhibition between aqueous and alcohol extracts against growth of *Aspergillus niger* at 50.0 g/ml concentrations

Concentration (g/ml)	**Group**	**Mean (SD)**	**Mean difference (95% CI)**	**t-statistic (df)**	**p-value**
50.0	Aqueous	14.00(0.71)	2.60(1.42,3.77)	5.10(8)	0.001
	Alcohol	11.40(0.89)			

Independent t-test was applied

Comparison on antifungal activity of kaffir lime leaf aqueous and alcohol extract against *Candida albicans*

Comparison between antifungal activity of KLL aqueous and alcohol extract against *Candida albicans* demonstrated that both alcohol and aqueous KLL extracts showed significant antifungal activities towards *Candida albicans* with widest zone of inhibition was 30 mm in alcohol extract. Antifungal activity in alcohol KLL extract is better than aqueous KLL extract. In each concentration of 50 g/ml, 25 g/ml and 12.5 g/ml, zone of inhibition alcohol extracts was more as compared to aqueous extracts (Table 8). Independent t-test showed that alcohol extracts has bigger mean of inhibition as compared to aqueous extract in all three concentrations with significant difference of $p<0.001$ against *Candida albicans*. Ninety five percent confidence interval of mean difference of inhibition does not include zero. Therefore, statistically antifungal activity of *Candida albicans* is better in alcohol KLL extract than aqueous extract.

Table 8: Comparison of mean of zone inhibition between aqueous and alcohol extracts against growth of *Candida albicans* at three different concentrations.

Concentration (g/ml)	**Group**	**Mean (SD)**	**Mean difference (95% CI)**	**t-statistic (df)**	**p-value**
50.0	Aqueous	27.20(0.84)	-2.80(-4.14,-1.46)	-4.80(8)	0.001
	Alcohol	30.00(1.00)			
25.0	Aqueous	17.20(0.84)	-6.20(-7.23,-5.17)	-13.86(8)	<0.001
	Alcohol	23.40(0.55)			
12.5	Aqueous	11.00(1.00)	-2.80(-4.14,-1.45)	-4.80(8)	0.001
	Alcohol	13.80(0.84)			

Independent t-test was applied

Comparison on antifungal activity of kaffir lime leaf between *Candida albicans* and *Aspergillus niger*

Comparison on antifungal activity of KLL between *Candida albicans* and *Aspergillus niger* were done using independent t-test (Table 9). *Candida albicans* was inhibited better as compared to *Aspergillus niger* in aqueous KLL extracts as shown in Table 3.9 with significant p<0.001 for all three concentrations and *Candida albicans also* was inhibited better as compared to *Aspergillus niger* in alcohol extract. Comparison with significant difference is shown in Table 10. Both ninety five percent confidence interval of mean difference of inhibition does not include zero.

Table 9: Comparison of mean of zone inhibition against *Candida albicans* and *Aspergillus niger* on aqueous Kaffir lime leaves extract at three different concentrations.

Concentration	**Group**	**Mean (SD)**	**Mean difference (95% CI)**	**t-statistic (df)**	**p-value**
50.0 g/ml	*Aspergillus niger*	13.80(3.77)	-13.40(-17.38,-9.41)	-7.76(8)	<0.001
	Candida albicans	27.20(0.84)			
25.0 g/ml	*Aspergillus niger*	0	-17.20(-18.06,-16.34)	-45.97(8)	<0.001
	Candida albicans	17.20(0.84)			
12.5 g/ml	*Aspergillus niger*	0	-11.00(-12.03,-9.97)	-42.60(8)	<0.001
	Candida albicans	11.00(1.00)			

Independent t-test was applied

Table 10: Comparison of mean of zone inhibition against *Candida albicans* and *Aspergillus niger* on alcohol Kaffir lime leaves extract at three different concentrations.

Concentration	**Group**	**Mean (SD)**	**Mean difference (95% CI)**	**t-statistic (df)**	**p-value**
50.0 g/ml	*Aspergillus niger*	11.40(0.89)	-18.60(-19.98,-17.22)	-31.00(8)	<0.001
	Candida albicans	30.00(1.00)			
25.0 g/ml	*Aspergillus niger*	0	-23.40(-23.96,-22.84)	-95.53(8)	<0.001
	Candida albicans	23.40(0.55)			
12.5 g/ml	*Aspergillus niger*	0	-13.80(-14.66,-12.94)	-36.88(8)	<0.001
	Candida albicans	13.80(0.84)			

Independent t-test was applied